Journal of Chromatography, 444 (1988) 115–122
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1360

APPLICATION OF MARFEY'S REAGENT IN RACEMIZATION STUDIES OF AMINO ACIDS AND PEPTIDES

GYULA SZÓKÁN*, GÁBOR MEZÖ and FERENC HUDECZ
Institute of Organic Chemistry, Eötvös University, Muzeum krt. 4/B, H-1088 Budapest (Hungary)

SUMMARY

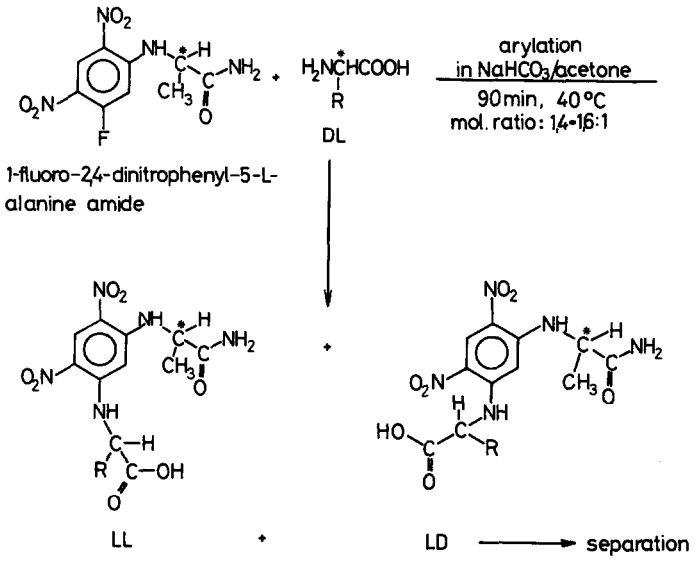
Reversed-phase high-performance liquid chromatography and pre-column derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) were used for monitoring racemization in peptides, amino acids and their derivatives by separation of optical isomers of amino acids. The technique was applied to the analysis of biologically active peptides, amino acids, their N- and C-protected derivatives, branched polypeptides based on polylysine, and endothiopeptides, and to the detection of stereochemical consequences of side reactions and hydrolysis. The chromatographic samples were mixtures of L- and D-amino acids obtained by hydrolysis of different peptides and derivatives. Baseline separations could be achieved on an ODS-Hypersil column with methanol–acetonitrile–acetate buffer (pH 4) mixtures as the eluents. The rates of racemization were calculated.

INTRODUCTION

Biologically active peptides should be very pure stereochemically. Their synthesis also requires optically pure amino acid derivatives. Therefore, the rapid and accurate determination of the optical purity of amino acids and peptides is topic of great importance. High-performance liquid chromatography (HPLC) is the method most frequently used for this purpose. For enantiomer resolution of chiral amino acids, direct techniques are applied, in which either chiral stationary phases or chiral additives to the mobile phase are used. Derivatization procedures with chiral reagents have also been developed to produce covalently bound diastereomeric derivatives. In these cases, ordinary stationary phases and eluents can be applied for chromatographic resolution. The chiral reagents used in amino acid analysis include 2,3,4,6-tetraacetyl-D-glycopyranosyl isothiocyanate[1], (−)-α-methoxy-α-methyl-1-naphthalene-acetic acid[2], (+)-1-(9-fluorenyl)ethyl chloroformate[3] and some amino acid derivatives, such as the N-protected cysteine and *o*-phthalaldehyde adduct[4], *tert.*-butoxycarbonyl-L-amino acid-*N*-hydroxysuccinimide esters[5], *N*-carboxy-L-leucine anhydride[6], D- or L-*O*-(4-nitrobenzyl)tyrosine methyl ester[7] and Marfey's reagent[8].

Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide reacts (Scheme 1), with the optical isomers of amino acids to form diastereomeric N-aryl derivatives which can be separated by conventional HPLC methods. The derivatives have an

0021-9673/88/$03.50



Scheme 1. Reaction of DL-amino acids with Marfey's reagent.

absorption maximum at 340 nm with an extinction coefficient of $\varepsilon \approx 3 \cdot 10^4$, and, therefore, they can be detected by UV spectroscopy with high sensitivity[8]. The separation method was demonstrated by Marfey for five amino acids (Ala, Asp, Glu, Met and Phe) on a C_{18} column with an acetonitrile gradient system[8].

The present paper describes the application of Marfey's reagent to the separation of amino acid enantiomers and to the study of epimerization of peptides. Marfey's method has been improved and extended to all the common protein amino acids and multicomponent amino acid mixtures.

EXPERIMENTAL

Materials

The peptides and amino acid derivatives investigated (Table I) were synthesized by the Research Group for Peptide Chemistry of the Hungarian Academy of Sciences, Budapest, the Institute of Organic Chemistry, Eötvös University, Budapest and the Chemical Works, G. Richter, Budapest. The abbreviations used follow the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature[12].

The separations were characterized by resolutions, R_s, and separation coefficients, α, calculated from the peak data for the diastereomeric amino acid derivatives.

The peptide samples and derivatives were hydrolyzed in 6 *M* hydrochloric acid at 105°C for 24 h in sealed tubes. The acid was removed *in vacuo*, and the acid-free hydrolysate was derivatized with Marfey's reagent.

Derivatization

Derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Pierce, Rockford, IL, U.S.A.) was carried out according to Marfey[8]. The hydrolysate

TABLE I

PEPTIDES AND AMINO ACID DERIVATIVES INVESTIGATED

Amino acid derivatives

BOC-Ala-OH HCl · Ala-OCH_3
Z-Ala-OH Z-Leu-OPcp

Peptides

Z-Phe–Leu-OCH_3
Z-Lys–Lys-OCH_3
| |
BOC BOC

Z-DL-Ala–DL-Ala–DL-Ala–Leu-OPcp
Z-DL-Ala–DL-Ala–DL-Ala–Leu-OCH_3
Z-DL-Ala–D-Ala–DL-Ala–Leu-OH

H-Ala–Pro-OH
H-Asp–Phe-OH
H-Arg–Lys–Asp-OH
cyclo(Alat–Alat)

Branched polypeptides[10,11]	*Code**	*Molar ratio of amino acid*			$\bar{M}_W$
		Lys	Ala_m	X**	
poly Lys-(DL-Ala_m–Leu_l)	ALK	1	3.0	0.7	38 900

* Based on one-letter symbols for amino acids: A = Ala; K = Lys; L = Leu.
** X = Leu.

prepared from 2.5 μmol of material was dissolved in 100 μl of 0.5 *M* sodium bicarbonate solution and 200 μl of a 1% solution of Marfey's reagent in acetone were added. The solution was incubated at 40°C for 90 min and, after cooling, 25 μl of 2 *M* hydrochloric acid were added. After 15–20-fold dilution in methanol or eluent, 10–20-μl aliquots were used for injection.

High-performance liquid chromatography

Separations were performed on a laboratory-assembled instrument consisting of a reciprocating piston pump (Model 1515; Orlita, Giessen, F.R.G.), a variable-wavelength UV monitor, fitted to an 8-μl flow-cell (Model 212; Cecil, Cambridge, U.K.) and a sample injector (Model 7011 loop injector; Rheodyne, Berkeley, CA, U.S.A.). Column effluents were monitored at 340 nm. The packing material was Hypersil ODS (Shandon Southern Products, Runcorn, U.K.). All solvemts were of HPLC grade, from Reanal (Hungary). The mobile phases are listed in Table II. The chromatograph was operated isocratically or with a methanol gradient at ambient temperature. The mobile phase flow-rates were between 0.8 and 1.5 ml/min. Peaks were recorded on a Model OH-814/1 chart recorder (Radelkis, Hungary).

RESULTS

Marfey's reagent allowed the resolution by HPLC of all optical isomers in amino

TABLE II

CHROMATOGRAPHIC DATA FOR MARFEY'S DERIVATIVES OF AMINO ACIDS

For comparison k', R_s and α values were also calculated for gradient elution.

Amino acid	k'		α	R_s	*Eluent system (v/v)*
	L	*D*			
Phe	7.9	29.5	4.1	7.3	Methanol–buffer (45:55)
Phe	36.2	43.8	1.2	7.6	Methanol gradient* in 80:20
Ala	2.1	6.1	2.9	8.0	Methanol–acetonitrile–buffer (20:10:70)
Leu	21.1	45.4	2.1	14.8	20:10:70
Lysα	5.1	7.2	–	–	20:10:70
ε	8.9	8.0	–	–	30:10:60
bis	30.3	36.0	–	–	(for D-derivatives)
Val	9.0	40.0	4.4	12.9	20:10:70
Thr	5.8	25.0	4.3	19.2	gradient*
Ser	5.6	10.0	1.7	6.2	Methanol–buffer (20:80)
His	11.7	13.8	1.1	11.3	Gradient
Met	8.0	34.5	4.3	17.6	20:10:70
Asp	1.4	3.7	2.6	4.0	20:5:75
Glu	1.8	4.2	2.3	3.4	20:10:70
Gly		8.1			Methanol gradient* in 80:20
Arg	9.8	15.4	1.5	2.7	20:10:70

* Eluents: A = 0.02 *M* sodium acetate buffer (pH 4)–methanol (4:1, v/v); flow-rate 1 ml/min; B = methanol; linear gradient of 1.5 ml B/min after 9 min.

acid mixtures after a simple derivatization procedure. The samples were obtained by hydrolysis of different peptides, proteins and amino acid derivatives. They could be simply and conveniently N-arylated with the reagent. The procedure was similar to the Sanger reaction, and the reaction was complete in 90 min. The diastereomeric N-aryl derivatives were separated and quantitated by reversed phase (RP)-HPLC on an ODS-Hypersil column. Chromatographic conditions were optimized to achieve large separation coefficients, α, higher resolutions, R_s (Table II), and baseline separations with methanol–sodium acetate buffer (pH 4.0) or methanol–acetonitrile–sodium acetate buffer eluent systems[9], either isocratically or by means of gradient elution. In most cases the separation of derivatives was achieved isocratically using ternary mixtures (Fig. 1). The determination of amino acid enantiomers in peptide or protein samples requires both adequate resolution of the enantiomers and good selectivity in the separation of different amino acids. The elution was optimal when performed isocratically for 8–10 min, followed by a linear gradient (Fig. 2). Some amino acids, such as Lys, Arg, Orn and His, gave more than one aryl derivative, which also were separable; *e.g.*, in the case of lysine, α-, ε- and bis(aryl)lysine derivatives were formed and separated isocratically (Fig. 1). By using three times as much reagent for the derivatization as did Marfey, only bis(aryl)lysine was identified on the chromatogram.

Chromatographic data for some amino acid derivatives are summarized in Table II. It seems that the L-diastereomers are usually eluted before the D-isomers, and ionizable side chains decrease the retention, whereas hydrophobic side chains increase it. The hydrolyzed reagent, 1-hydroxy-2,4-dinitrophenyl-5-L-alanine amide (Marfey-

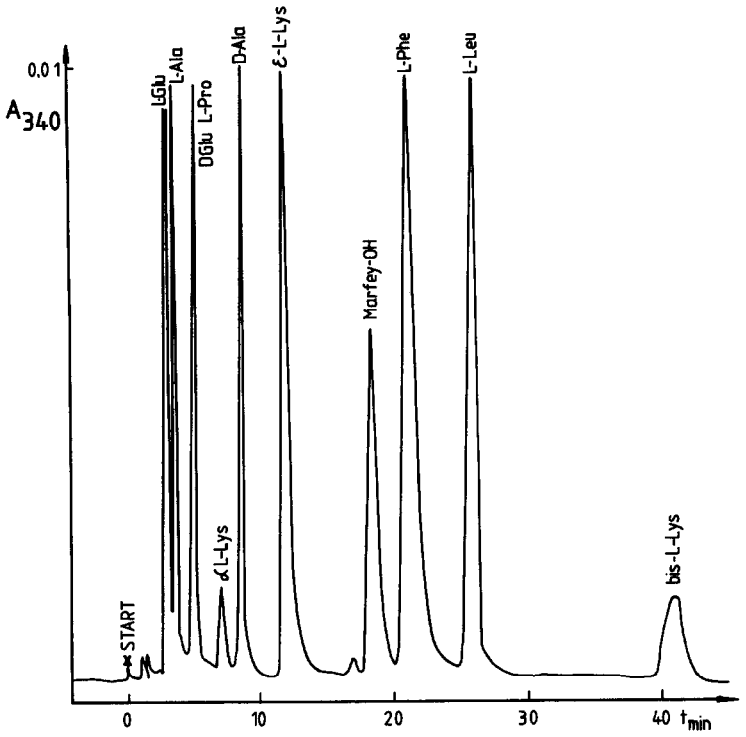


Fig. 1. Isocratic separation of some DL-amino acid derivatives. Column: Hypersil ODS-6 (125 mm × 4 mm). Eluent: methanol–0.02 *M* sodium acetate buffer (pH 4)–acetonitrile (2:7:1, v/v/v); flow-rate 1.04 ml/min. Detection: 340 nm. Sample injected: 10 μl containing 100 pmol per amino acid.

OH) always produces an extra peak. Its capacity factor is 15.1 with the 20:10:70 eluent and 30.7 with the 10:10:80 eluent.

Racemization of amino acids can be determined by measuring quantitatively the D-amino acid content of the sample, or directly the D/L amino acid derivative ratio. Thus, the optical purities of amino acids used in peptide syntheses were checked very easily by our method; *e.g.*, in the commercial L-aspartic acid standard we found 0.10% of the D-enantiomer. The detection limit was 5–8 pmol of amino acid enantiomer in our HPLC system. There was no detectable D-Ala in the standard L-Ala samples, so it may be concluded that racemization during derivatization is negligible.

Since racemization (epimerization) is one of the side reactions commonly occurring in peptide syntheses[13,15], the HPLC methods described above were applied to determine its degree. In the case of peptides a strategy involving hydrolysis (in 6 *M* hydrochloric acid, derivatization (with Marfey's reagent) and HPLC separation of diastereomeric amino acid derivatives (on an ODS-Hypersil column) was applied. The risks involved here include the possibility of racemization during hydrolysis, too. For example, 11.8% of D-Asp was measured by isocratic HPLC with the ternary system 20:5:75 (see Table II) in L-Asp after treatment wth 6 *M* hydrochloric acid for 24 h.

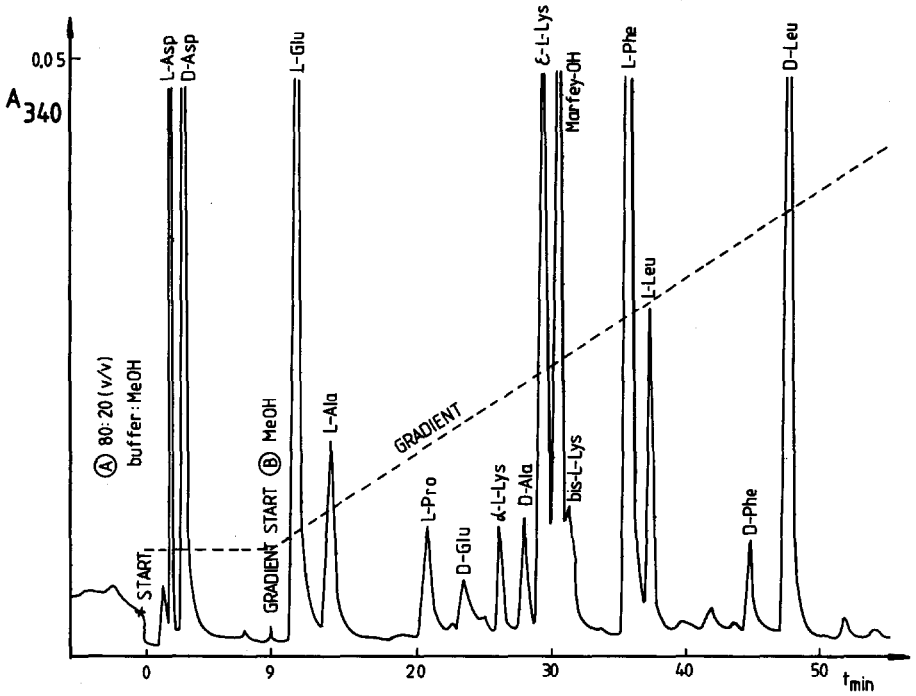


Fig. 2. Separation of amino acids by gradient elution. Column: Hypersil ODS-6 (125 mm × 4 mm). Eluents: A = 0.02 *M* sodium acetate buffer (pH 4)–methanol (4:1, v/v); flow-rate 1 ml/min; B = methanol; linear gradient after 9 min of 1.5 ml (B)/min. Detection: 340 nm. Sample injected: 10 μl containing 50–600 pmol of L- and D-amino acids.

TABLE III

D-Leu CONTENT IN PEPTIDE ACTIVE ESTERS AND POLYPEPTIDES

Abbreviations: DCC = dicyclohexylcarbodiimide; HOPcp = pentachlorophenol; THF = tetrahydrofuran; DMF = dimethylformamide; TEA = triethylamine; NMM = *N*-methylmorpholine; HOBt = 1-hydroxybenzotriazole.

Z-DL-Ala-DL-Ala-DL-Ala-Leu-OPcp			*poly-Lys-(DL-Ala-DL-Ala-DL-Ala-Leu)*		
Preparation method[15]		D-*Leu* (%)	*Coupling conditions*		D-*Leu* (%)
Coupling	*Solvent*		*Solvent, base*	*v/v*	
DCC–HOPcp (1:1)	THF	15.8	DMF–water TEA	9.1	28.9
DCC–HOPcp (1:3 complex)	Ethyl acetate	13.0	DMF–water NMM, HOBt	9:1	21.3
"Backing off" procedure[14]	DMF	4.7	DMF–water TEA	9:1	17.6

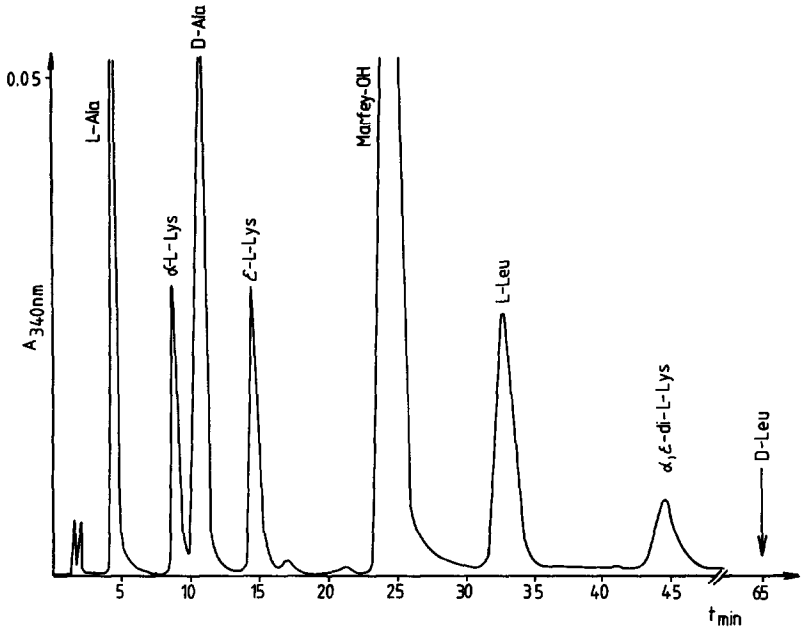


Fig. 3. Racemization studies on the branched polypeptide ALK. Separation of L- and D-amino acids from ALK hydrolysate. Column: Hypersil ODS-6 (125 mm × 4 mm). Eluent: methanol–0.02 *M* sodium acetate buffer (pH 4)–acetonitrile (2:7:1, v/v/v); flow-rate 0.8 ml/min. Detection: 340 nm. Sample preparation: 1.8 mg of polypeptide were hydrolyzed and derivatized as described; after twenty-fold dilution in methanol, 10-μl aliquots were injected.

Examples of application

(1) In the preparation of LL-Ala-Pro dipeptide with a new reagent, 49.9% of D-Ala was found (eluent 20:10:70), indicating complete racemization during peptide coupling.

(2) Stereochemical consequences of side reactions were analyzed, *e.g.*, trans-peptidation caused 5.6% racemization in Asp-Phe dipeptide. The L-Asp derivative showed very low retention on an ODS-Hypersil column with the 20:5:75 eluent.

(3) The hydrolysis products of peptide methyl esters with strong bases were also found to have some D-amino acid content (see data for Leu tetrapeptide in Table III).

(4) The optical purity of some synthetic biologically active peptides was determined. Some synthetic thymopoietin fragments, *e.g.*, LLL-Arg-Lys-Asp, contained a diastereomeric peptide impurity (34.6%), which was determined from the D-Asp content of the preparation by HPLC with the 20:5:75 isocratic system.

(5) From the determination of racemization rates of starting amino acid derivatives, BOC-amino acids prepared from di-*tert.*-butyl pyrocarbonate and *Z*-alanine did not exhibit any racemization, except when the starting L-amino acids, *e.g.*, Asp and Glu contained 0.1–0.8% of the D-enantiomers.

(6) The enantiomer composition of side chains in branched polypeptides was investigated systematically. Data for high-molecular-weight multichain polypeptides containing a poly-α-L-lysine backbone substituted at the ε-amino groups with side chains comprised of about three DL-Ala residues and another amino acid residue[10,11]

(either Leu, Glu or Phe) were measured. In the case of the ALK polypeptide (see Experimental) the separation of L- and D-amino acids was achieved isocratically with the 20:10:70 eluent system (Fig. 3). 50.4% of L-Ala and 49.6% of D-Ala were measured, indicating that no stereospecific or stereoselective polymerization took place and the coupling of D- or L-amino acid to the Ala backbone did not result in racemization.

(7) D/L amino acid ratios were measured after acidic hydrolysis in endothiopeptides[16], *e.g.*, in cyclo(Alat-Alat), a D/L ratio of 37:63 was calculated from the baseline resolution of DL-Ala in the 20:10:70 eluent system.

(8) Using our method with the 20:10:70 eluent, the degree of racemization was determined during the preparation of some peptide active esters, *e.g.*, the D-Leu content was measured in benzyloxycarbonyl-DL-Ala-DL-Ala-D-Ala-Leu pentachlorophenyl ester prepared by two different methods (Table III). During the synthesis with the carbodiimide–pentachlorophenol procedure from the N-protected tetrapeptides prepared by hydrolysis of the peptide methyl ester, 13–16% of D-Leu was formed, whereas the "backing off" procedure[14] showed only 4.7% (Table III).

(9) The coupling of benzyloxycarbonyl-DL-Ala-DL-Ala-DL-Ala-Leu pentachlorophenyl ester to a polypeptide, as poly-L-lysine, was also monitored in the same way. In this case the coupling led to high racemization: 17–29% of D-Leu was measured.

According to our experience, Marfey's method when improved and extended as described can also be applied to the screening of D-amino acids in antibiotic compounds, in bacterial cell walls, in fossil shells or bones and in other molecules of biological origin.

ACKNOWLEDGEMENT

The authors thank Mrs. Almás for help with the HPLC analyses.

REFERENCES

1 N. Nimura, H. Ogura and T. Kinoshita, *J. Chromatogr.*, 202 (1980) 375.
2 J. Goto, M. Hasegawa, S. Nakamura, K. Shimada and T. Nambara, *J. Chromatogr.*, 152 (1978) 413.
3 S. Einarsson, B. Josefsson, P. Möller and D. Sanchez, *Anal. Chem.*, 59 (1987) 1191.
4 R. H. Buck and K. Krummen, *J. Chromatogr.*, 315 (1984) 279.
5 A. R. Mitchell, S. B. H. Kent, I. C. Chu and R. B. Merrifield, *Anal. Chem.*, 50 (1978) 637.
6 T. Takaya, Y. Kishida and S. Sakakibara, *J. Chromatogr.*, 215 (1981) 279.
7 S. Görög, B. Herényi and M. Löw, *J. Chromatogr.*, 353 (1986) 417.
8 P. Marfey, *Carlsberg Res. Commun.*, 49 (1984) 591.
9 Gy. Szókán, A. Török and B. Penke, *J. Chromatogr.*, 387 (1987) 267.
10 F. Hudecz and M. Szekerke, *Collect. Czech. Chem. Commun.*, 45 (1980) 933.
11 F. Hudecz and M. Szekerke, *Collect. Czech. Chem. Commun.*, 50 (1985) 103.
12 IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, 247 (1972) 977.
13 D. S. Kemp, in E. Gross and J. Meienhofer (Editors), *The Peptides*, Vol. 1, Academic Press, New York, 1977, p. 315
14 M. Goodman and K. C. Stueben, *J. Am. Chem. Soc.*, 81 (1959) 3980.
15 M. Bodansky, in E. Gross and J. Meienhofer (Editors), *The Peptides*, Vol. 1, Academic Press, New York, 1983, p. 112.
16 K. Clausen, M. Thorsen and S.-O. Lawesson, *Tetrahedron*, 37 (1981) 3635.